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# PerKit™ Antibody Methotrexate Conjugation Kit (CM11407 and CM11407x3) User Reference Guide

## Contents

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# Important Notes & Contact Information

**READ BEFORE USING ANY RESOURCES PROVIDED HEREIN**

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Sample data are provided for illustration and example purposes only and represent a small dataset used to verify kit performance in the CellMosaic laboratory. Information about the chemicals and reagents used in the kit are provided as necessary.

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## Kit Components

This kit provides materials to conjugate 1 to 3 mg of antibody samples (**IgG**) with methotrexate.

⚠ Upon receipt, please remove **Box 1** and store in a freezer at or below -20°C.
Store **Box 2** in a refrigerator at 2-8°C.

| | Name | Part # | Quantity (CM11407) | Quantity (CM11407x3) | Storage condition |
|---|---|---|---|---|---|
| **Box 1** | Methotrexate NHS ester (dark red label) | CM11009.1 | 1 unit | 3 units | -20°C, dry |
| **Box 2** | Solution A (blue label) | CM01006 | 0.5 mL | 0.5 mL | 2-8°C |
| | Buffer A (orange label) | CM02001 | 4 mL | 12 mL | |
| | Storage Buffer (1 x PBS buffer) (grey label) | CM02013 | 20 mL | 60 mL | |
| | ADC Stabilizing PBS Buffer (5x) (pink label) | CM02022 | 0.5 mL | 1.5 mL | |
| | Centrifugal Filter Device | CM03CD050A | 2 | 6 | |
| | Desalting Column | CM03SG10 | 1 | 3 | |
| | Collection Tubes | N/A | 4 | 12 | |
| | 1.5 mL Centrifuge Tube | N/A | 1 | 3 | |
| | 2.0 mL Centrifuge Tube | N/A | 1 | 3 | |
| | Hazardous Waste Bag | N/A | 1 | 3 | |
| User Material | IgG Antibody | N/A | NOT PROVIDED (User Supplied Material, **1-3 mg IgG** needed per reaction) | | |

**Reaction Scale:** The protocol is optimized for conjugating 3 mg of IgG antibody. If you have less than 3 mg of IgG, use the calculations in **Steps B10**, **C2**, **D5,** and **D6** to obtain the correct volumes to be added in each step.

## Safety Information

Warning: some of the chemicals used can be potentially hazardous and can cause injury or illness. Please read and understand the Safety Data Sheets (SDS) available at CellMosaic.com before you store, handle, or use any of the materials.

## Labeling Chemistry

The kit is designed to label any antibody (IgG type) with methotrexate directly via one of its carboxylic acid groups. The user supplies the antibody. This kit includes methotrexate NHS ester, which can be coupled to the antibody directly via surface amines in a single step. The product is then purified to remove any unreacted drug.

Key features of this conjugation kit:

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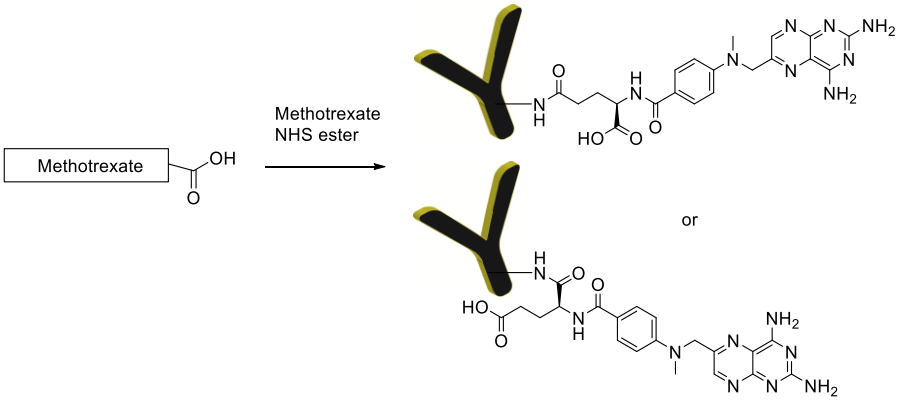
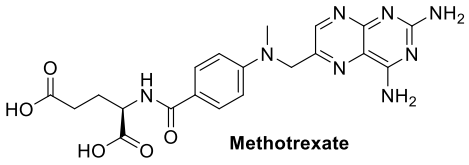
- Offers a simple and easy way to label IgG with methotrexate with minimum exposure to the chemotherapeutic drug
- Stable linkage
- Fast and easy preparation: 6 h preparation with <1 h hands-on time
- All reagents and supplies included for preparation and purification
- DAR with average 3 to 5 methotrexates per antibody
- Included stabilizing buffer for long-term storage
- >99% conjugated products by size-exclusion chromatography (SEC) and free of any unreacted drugs




**Drug Information:**




- **Name**: Methotrexate (amethopterin)
- **CAS number**: 59-05-2
- **Chemical Formula**: $C_{20}H_{22}N_8O_5$
- **MW**: 454.45 Da
- **Mechanism of action**: Competitively inhibits dihydrofolate reductase (DHFR), an enzyme that participates in tetrahydrofolate synthesis. Folic acid is essential for de novo synthesis of the nucleoside thymidine. In addition, folate is essential for biosynthesis of purine and pyrimidine bases. By inhibiting DHFR, methotrexate inhibits the synthesis of DNA, RNA, thymidylates, and proteins.
- **Medical usage**: Breast cancer, leukemia, lung cancer, lymphoma, and osteosarcoma

**Requirements for antibody (IgG):**

1. Preferably > 90% pure by gel electrophoresis

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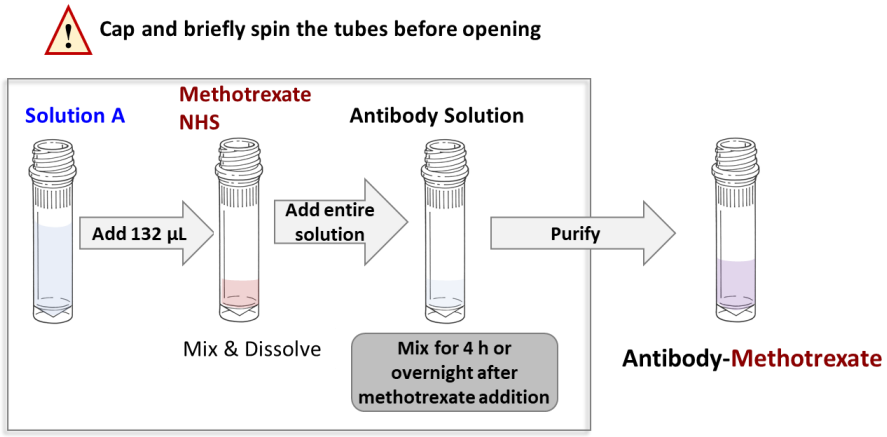
2. Total amount: 1-3 mg protein content as measured by UV. Note: the accuracy of your protein amount is the single most important factor to obtaining an optimized DAR. Please refer to the section Other Considerations in this manual to measure the protein amount

## Support

Customer can request a recommendation for conjugation if the molecule has a special feature or if less than 1 mg of antibody. CellMosaic also provides additional support services to customers who need help analyzing the final conjugates by HPLC.

## Protocol




**Scheme 1**. Schematic diagram of the workflow for preparing antibody-methotrexate conjugates starting with 3 mg of IgG (volume of reagents varies if the amount of IgG is < 3 mg)

### 1. Lab Instrumentation Needed

- Vortex mixer, centrifuge (preferably refrigerated, 14,000 g capable), mini-centrifuge
- Pipettes and tips
- Timer
- Incubator or shaker set at 25°C or RT
- Chemical hood
- Support stand, lab frame, or any support rod for desalting column
- Flask
- Personal protection equipment (lab coat, safety glasses, and chemical resistant nitrile gloves)

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## 2. Prepare Site and Reagents for Labeling Experiment

> **Note:** It is recommended that the labeling experiment be planned a few days before your other experiments. For long-term storage, please use the stabilization PBS buffer to store under recommended conditions.

Ensure you use personal protection equipment (lab coat, safety glasses, and chemical resistant nitrile gloves) while handling methotrexate. Locate a clean space inside a chemical hood.

**A1**. Remove **Box 1** containing **methotrexate NHS ester** (dark red label) from the -20°C freezer and warm to RT before opening the bag.
**A2**. Remove **Box 2** from the refrigerator. Take the hazardous waste bag and place it inside the chemical hood for solid waste disposal. Bring the rest of the items to a lab bench.
**A3**. Briefly spin the centrifuge tube containing **methotrexate NHS ester**. Place the **methotrexate NHS ester** tube in a tube holder inside a chemical hood and wait until the antibody is ready for conjugation.
**A4.** Set the temperature of the incubator or shaker to 25°C.

## 3. Preparation of Antibody Samples for Conjugation

Items needed: Filter Devices (CM03CD050A), Collection Tubes, Buffer A (CM02001, Orange label), 1.5 mL Centrifuge Tube, Clean Centrifuge Tubes (not provided in the kit).

Total amount of antibody used for the conjugation is 3 mg (protein content measured by UV) per reaction.

> **Reaction Scale:** If you have less than 3 mg of antibody, use the calculations in **Steps B10**, **C2**, **D5,** and **D6** to obtain the correct volumes to be added in each step.

**B1**. Insert the **Filter Device** into one of the provided collection tubes (microcentrifuge tube with the cap attached). Perform the step based on the following conditions.

- ✓ If your antibody is supplied as a lyophilized solid, dissolve the antibody in 500 µL of **deionized water** and then transfer the entire contents to the **Filter Device**.
- ✓ If your antibody is supplied in < 500 µL of buffer, transfer your antibody sample to the **Filter Device** directly. Add **Buffer A** to make up the total volume to 500 µL and cap it.
- ✓ If the volume of your antibody sample is between 500 and 1000 µL, divide the volume into two **Centrifugal Filter Devices**. Add **Buffer A** to make up the total volume in each filter device to 500 µL and cap them.
- ✓ If the volume of your antibody sample is >1000 µL, add up to 500 µL of sample to each of the two **Filter Devices** and cap them. Repeat Steps **B1-B4** until all of the antibody sample goes into the **Filter Device**. Move on to Step **B5**. Add **Buffer A** to make up the total volume to 500 µL in each device for the last refill.

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**B2**. Place the capped **Filter Device** into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device.

**B3**. Spin the **Filter Device** at 14,000 x g for 8 minutes (preferably cooled to 4°C) to concentrate to < 100 µL. (Spin time depends on many factors. The typical spin time for a 500 µL sample is approximately 8 to 20 minutes. The typical volume is ~40 µL after spinning for 8 minutes in an Eppendorf 5417R at 4°C).

**B4**. Remove the assembled device from the centrifuge and separate the **Filter Device** from the collection tube. Transfer the filtrate from the collection tube to a clean centrifuge tube (not provided). **Save the filtrate until the experiments are done.**

**B5**. Insert the **Filter Device** back into the collection tube. Add 400-450 µL of **Buffer A** to make up the total volume to 500 µL. Then place the capped **Filter Device** into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device. Spin the device at 14,000 x g to concentrate to < 100 µL. Remove the assembled device from the centrifuge and separate the **Filter Device** from the collection tube. Transfer the filtrate from the collection tube to a clean centrifuge tube (not provided). **Save the filtrate until the experiments are done.**

**B6**. Repeat **Step B5** two more times.

**B7**. Transfer the concentrated sample from the **Filter Device** to a 1.5 mL micro-centrifuge tube (use the Pipetman to estimate the approximate volume of the concentrated sample).

**B8**. Add 100 µL of **Buffer A** to the **Filter Device** to rinse. Stir it gently with a pipette tip, then transfer the entire contents to the 1.5 mL micro-centrifuge tube from **Step B7.**

**B9.** Repeat **Step B8** once.

**B10**. Add **Buffer A** to the 1. 5 mL micro-centrifuge tube from **Step B9** to make up the total volume of the sample to **618 ± 5 µL** and cap it.

**Calculation 1 for Less Antibody (Ab)**:

$$Total\ volume\ of\ the\ antibody\ in\ Step\ \boldsymbol{B10}\ (\mu L) = Ab\ in\ mg\ \times 206$$

**B11**. Vortex the combined antibody sample for 30 seconds and spin down.

## 4. Methotrexate Labeling (Step 1 in Scheme 1)

**C1**. Spin **Solution A** (blue label) to ensure there is no liquid in the cap before opening it. Add **132 µL** of **Solution A** to the **methotrexate NHS ester** tube from **Step A3**. Vortex for 30 seconds to 1 minute to dissolve the reagent and then spin down.

**Tip for solubility check**: Check the bottom of the micro-centrifuge tube to ensure the solution is clear of any solid residue.

**C2.** Transfer the entire **methotrexate NHS ester solution** from **Step C1** to the antibody solution from **Step B11**. When you add the methotrexate solution (MTX), place the pipette tip inside the

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antibody solution and then dispense the methotrexate slowly by stirring with the pipette tip. **Dispose of the pipette tip and methotrexate tube in the solid waste bag**.

**Calculation 2 for Less Antibody (Ab)**:

$$Volume\ of\ methotrexate\ solution\ to\ be\ transferred\ in\ Step\ \boldsymbol{C2}\ (\mu L) = Ab\ in\ mg\ \times 44$$

**C3**. Cap the centrifuge tube. Mix at 25°C or RT for 4 h or overnight (less than 16 h).

**Tip for mixing**: You can use a nutator, a shaker, vortex, or an incubator shaker for mixing. If you are using end to end nutating, make sure your centrifuge is capped properly. If you don't have any of this equipment, you can let the centrifuge tube sit at the bench with manual mixing by pipetting every 20 minutes.

**Time-saving tip**: While waiting for the reaction to complete, you can move on to **Step D1** and equilibrate the column for purification.

## 5. Purification of Conjugate

Items needed: Desalting Column (CM03SG10), Storage Buffer (1x PBS, CM02013, grey label), 2.0 mL Centrifuge Tube, Hazardous Waste Bag, Antibody Solution from **Step C3**.

**D1.** In a chemical hood, securely attach the **Desalting Column** to a support stand, lab frame, or any support rod. Remove the top and bottom caps from the column and allow the excess liquid to flow through by gravity. Collect the liquid in a flask.
**D2.** Add 5 mL of **PBS buffer** and allow the buffer to completely enter the gel bed by gravity flow.
**D3.** Repeat **Step D2** twice.
**D4.** Spin the methotrexate-labeled antibody solution from **Step C3** to ensure there is no liquid in the cap before opening it. Add the entire antibody solution to the column. Allow the sample to enter the gel bed completely. **Dispose of the centrifuge tube in the solid waste bag.**
**D5.** Add 250 µL of **PBS buffer** and allow the liquid to enter the gel bed completely (**note:** this elute buffer does not contain any of your product, you can let it drain to the waste).

**Calculation 5 for Less Antibody (Ab)**:

$$Volume\ of\ Storage\ buffer\ in\ Step\ \boldsymbol{D5}\ (\mu L) = 1000 - Ab\ in\ mg\ \times 250$$

**D6.** Place a 2.0 mL centrifuge tube under the column. Add 1250 µL of PBS buffer to the column. Collect the eluent by gravity and allow the buffer to enter the gel bed completely.

**Calculation 6 for Less Antibody (Ab)**:

$$Volume\ of\ Storage\ buffer\ in\ Step\ \boldsymbol{D6}\ (\mu L) = 500 + Ab\ in\ mg\ \times 250$$

**D7**. Label the tube as your product. Store your conjugate at 4°C. **Dispose of the Desalting Column in the solid waste bag and seal the bag. Dispose of the waste following regulations appropriate for your area.**

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**D8.** Determine the concentration and estimated DAR by UV/Vis spectrophotometry (see other considerations).

**D9.** If the ADC is not used within a few weeks for the experiment, add **Stabilization PBS buffer (5x)** (pink label) to the ADC from **Step D7**. If the total volume of your ADC is 1.25 mL, you need to add 312.5 µL of Stabilization PBS buffer. Aliquot and store the conjugate in a freezer at less than -20°C or lyophilize to dryness for long-term storage.

## Conjugate is Ready for Your Experiment

- **Specification for your product:** Methotrexate-labeled antibodies with an average drug-to-antibody ratio (DAR) of 3-5. A typical batch contains >99% conjugated products by SEC and is free of any unreacted drug. The approximate concentration of the ADC is 1.44 mg/mL in PBS buffer assuming 60% recovery (without ADC stabilizing buffer).

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## Other Considerations

### 1. Concentration Determination for IgG Antibody (Unlabeled)

The accuracy of the IgG amount is important for obtaining an optimized DAR in this protocol. The simplest assay method for determining IgG concentration in solution is to measure the absorbance of the IgG at 280 nm (UV range) ($A_{1\ mg/mL}$ = 1.4).

If your antibody comes with a buffer that has no UV absorbance at 280 nm, you can measure the UV absorbance prior to starting an experiment.

$$Concentration\ (mg/mL)\ of\ IgG = \frac{(A280)}{1.4}$$

If your antibody comes with a buffer that has UV absorbance at 280 nm, you can determine the concentration in **step B11** after exchanging it with Buffer A and assuming **95%** recovery of the IgG after buffer exchange. Buffer A does not contain any substances that will interfere with the UV measurement at 280 nm. The total volume of Buffer A added in **Step B10** can be estimated based on the initially estimated amount of antibody and will not affect the conjugation too much if the volume is off to some extent.

$$Concentration\ (mg/mL)\ of\ Starting\ IgG = \frac{(A280)}{1.4 \times 0.95}$$

After calculating the total amount, follow the calculations in **Steps B10, C3**, **D9**, **E2**, **F5,** and **F6** to obtain the correct volumes to be added in each step.

### 2. Concentration Determination for ADC

To determine the concentration, dilute your conjugate from **Step D7** with 1x PBS buffer. Measure the UV absorbance of the conjugate at 280 nm (A280) using a UV spectrometer and calculate the concentration based on the following formula:

$$Concentration\ (\mu M)\ of\ the\ dilute\ sample = \frac{(A280) \times 1{,}000{,}000}{L\ (210{,}000 + n \times 17{,}224)}$$

$$Concentration\ (mg/mL)\ of\ the\ dilute\ sample = \frac{(A280) \times 150{,}000}{L(210{,}000 + n \times 17{,}224)}$$

Where **L** is the UV cell path length (cm). If you are using a 1 cm UV cell, you can dilute the conjugate 4 times to obtain a good reading.

Where **n** is the average molar ratio of methotrexate per antibody. Use 4.0 if you do not know the experimental value of your conjugates.

For a typical IgG with a MW of 150,000 Da, the molar extinction coefficient is 210,000 $M^{-1}cm^{-1}$. The molar extinction coefficient for methotrexate is 6100 $M^{-1}cm^{-1}$ based on CellMosaic's experimental data.

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## 3. MW Calculation

Calculation of the MW of the conjugate:

**MW(ADC) = n x 436.5 + 150,000**

Where **n** is the average molar ratio of methotrexate per antibody. Use **4.0** if you do not know the experimental value of your conjugates.

## 4. Drug-to-Antibody Ratio (DAR) and Characterization by UV and HPLC

In this kit, the target DAR is 3-5.

To estimate the DAR, you can obtain the UV absorbance ratio (R) of your conjugate at 302 nm and 280 nm.

$$R = {}^{(A302)}\Big/_{(A280)}$$

The unlabeled antibody will have an R value of 0.085. A methotrexate-ADC with a DAR of 3 – 5 will have an R value of 0.31– 0.41.

You can also use the following formula to calculate the estimated DAR (for reference only):

$$DAR = \frac{(13.29 \times R - 1.13)}{(1.28 - R)}$$

**Methotrexate**: $E_{280\ nm}$ = 17,224 $M^{-1}cm^{-1}$ and $E_{302\ nm}$ = 20,268 $M^{-1}cm^{-1}$ in PBS buffer according to data obtained from CellMosaic and based on the literature data (Touchette N.A.; Perry K. M.; Matthews, C. R. (1986) Folding of Dihydrofolate Reductase from *Escherichia coli*. *Biochemistry* **25**, 5445-5462. Dawson, R. M. C., Elliot, D. C., Elliot, W. M., & Jones, K. M. (1969) in Data for Biochemical Research, p 206, Oxford University Press, Oxford. $E_{302\ nm}$ = 22,100 $M^{-1}cm^{-1}$ in 0.1N NaOH).
**Antibody**: $E_{280\ nm}$ = 210,000 $M^{-1}cm^{-1}$ and 17,904 $M^{-1}cm^{-1}$ at 302 nm (data obtained from CellMosaic)

## 5. Characterization of ADC by HIC HPLC

For ADCs prepared via surface amines of the antibody, hydrophobic interaction chromatography (HIC) HPLC can be used to check if an antibody is labeled or not. However, due to the highly heterogeneous nature of surface amine labeling, antibody loaded with the same number of drugs (same DAR) may have slightly different hydrophobicity. For a typical SN38 ADC, a broad peak will be seen without clear separation of the peaks.

CellMosaic offers an HIC buffer set (Product #: CM02025) for our customers to use with any HIC column. The CM02025 product sheet contains all of the information and methodology needed to run an HIC HPLC analysis.

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If you do not have access to an HPLC facility, you can send your sample to CellMosaic for analysis.

## 6. Aggregation and Precipitation Issue and Characterization by SEC HPLC

Methotrexate is not considered very hydrophobic due to its two carboxylic acid groups. We have not seen any aggregations of the ADC caused by methotrexate labeling. However, we have observed lower recovery for ADC with higher loading compared to lower loading. You can use size-exclusion chromatography (SEC) to check the extent of aggregation. SEC separates the conjugates by apparent molecular weight (MW) or size in aqueous solution. The larger the MW of the conjugate, the earlier it elutes. By comparing the SEC profile of unlabeled IgG and the ADC, you can estimate how much aggregation is in the ADC.

CellMosaic offers two SEC standards (Product #: CM92004 and CM92005) for our customers to use with any SEC column. The CM92004 product sheet contains all of the information and methodology you need to run an SEC HPLC analysis.

If you do not have access to an HPLC facility, you can send your sample to CellMosaic for analysis.

## 7. ADC Stabilizing Buffer

CellMosaic's proprietary ADC stabilizing PBS buffer (5x) contains 5x PBS buffer and other stabilizers to prevent the hydrophobic drugs from interacting with each other during storage and causing the ADCs to precipitate out. Stabilization buffer also helps preserve the structure of the ADCs during lyophilization. The buffer is biocompatible and can be used directly for any *in vitro* and *in vivo* studies. For more information on the stabilization buffers, please check our website.

## 8. Recommended Storage Conditions

ADC labeled with methotrexate is relatively stable. Recommended use is within 2 weeks if you store methotrexate-ADC at 2-8°C. Based on our preliminary data, the conjugate made with this kit can remain stable in PBS buffer for a few days at 2-8°C (no long-term stability data). The stability of your conjugate may be different due to your antibody and should be checked by either HPLC or UV. If you need to store the ADCs for longer, please dilute your ADC in Stabilization PBS buffer (5x). Aliquot and store the conjugate in a freezer less than -20°C or lyophilize to dryness. Avoid repeated freeze and thaw cycles.

## 9. Submit Samples for HPLC Analysis

If you are submitting samples to CellMosaic for SEC and HIC analysis, please follow these instructions:

1) Go online: https://www.cellmosaic.com/hplc-analysis/, select SEC HPLC Analysis (Product# AS0023) and HIC HPLC Analysis (Product#: AS0025), choose the quantity (number of

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samples. Bulk discounts available for multiple samples) and submit the order. Alternatively, you can email info@cellmosaic.com for a quote and to place the order.

2) Dilute your un-conjugated antibody to 1 mg/mL in PBS buffer, then transfer 50 µL of the diluted solution to a 500 µL microcentrifuge tube. Label the vial properly.
3) Transfer 50 µL of ADC (non-diluted solution) to a 500 µL microcentrifuge tube and label the vial properly.
4) Ship your samples with a cold pack for overnight delivery.

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## Appendix: Typical Kit Performance Data (LC analysis, CellMosaic)

**Antibody information**: A therapeutic antibody (human IgG1 subtype)

**Lot number:** S283.S9.042320

**Figure 1**: SEC HPLC analysis of purified methotrexate-ADC (MTX-ADC) in PBS ADC stabilizing buffer. Two labeling reactions. Scale of the reaction: 0.5 mg of antibody each reaction. Not much aggregation was observed for high and low loading MTX-ADC.

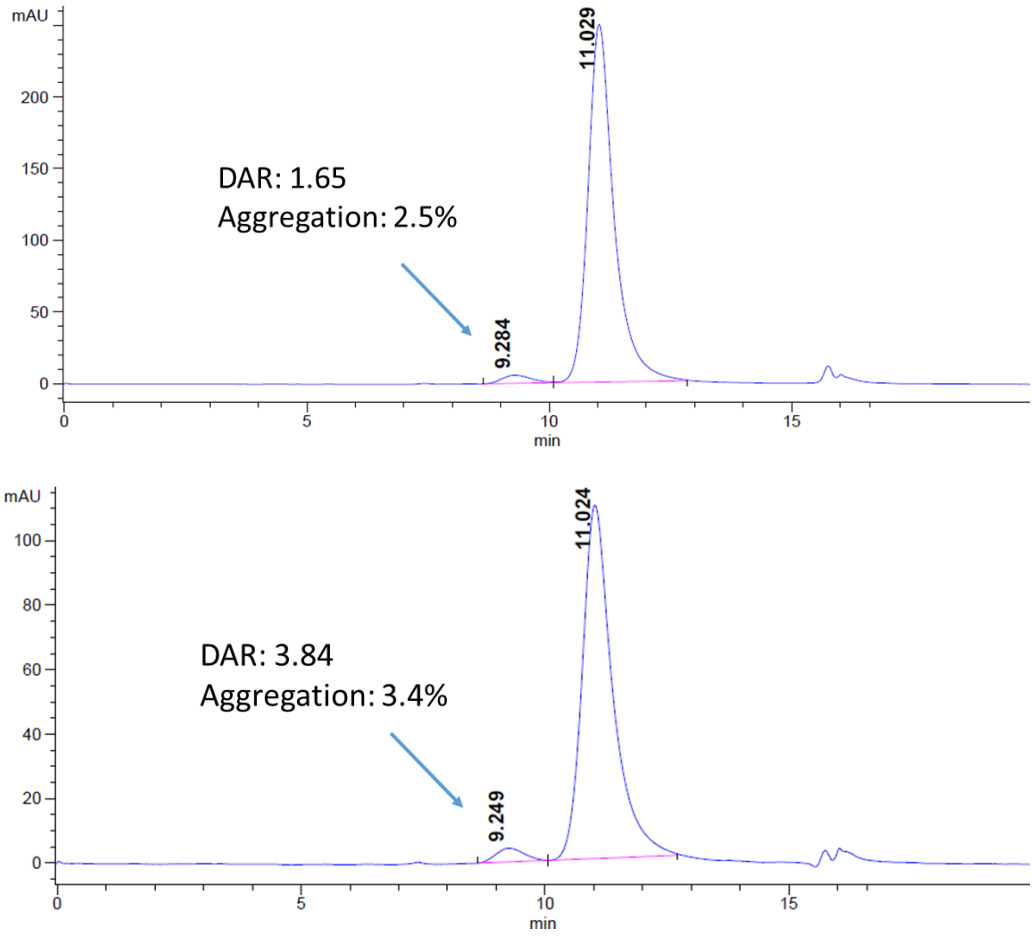

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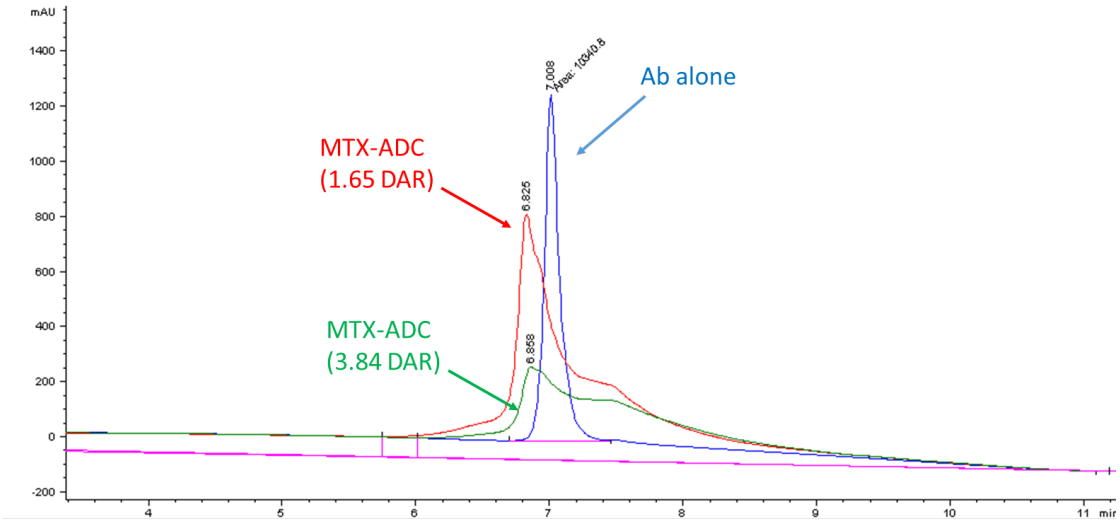
**Figure 2**: Overlay of HIC HPLC analysis of unlabeled antibody, MTX-ADC with DAR of 1.65, and MTX-ADC with DAR of 3.84.




**Summary of the results:**

| **R** value (consider the total peaks) | 0.217 | 0.353 |
|---|---|---|
| Average DAR based on R value | 1.65 | 3.84 |
| Extent of antibody aggregation (%) | 2.5 | 3.4 |
| Unreacted antibody (%) | N/A | 0 |
| Unreacted methotrexate (%) | 0 | 0 |
| Recovery (%) | 91 | 58 |